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Patterning of Cells on *Bioresist* for Tissue Engineering Applications

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INTRODUCTION

Engineering functional tissues and organs successfully depends on the ability to control cell orientation and distribution. Materials used for such purposes therefore have to be designed to facilitate cell distribution and eventually guide tissue regeneration in 3D. The field of tissue engineering hinges on developing degradable polymeric scaffolds that promote cell proliferation and expression of desired physiological behaviors through careful control of the polymer surface.

The development of materials for tissue engineering and guided tissue regeneration has accelerated over the last decade.[1] It has been demonstrated that non-patterned cells are effectively not tissue. "Tissues require that cells be placed and hold precise places often with precise orientations" [2-3]. Cell patterning is therefore very important for tissue engineering. We have developed a biocompatible, biostable chemically amplified *bioresist*, with which patterns are generated without involving harsh chemical treatment.

Combinatorial approach of polymer synthesis can be used to increase the number of available polymeric materials for any application and also to study the correlation s between polymer structures, material properties, and function [4].

In this paper, we present a combinatorial approach for the synthesis, characterization and cell patterning using the copolymers, 3-(*tert*-Butoxycarbonyl)-N-Vinyl-2-Pyrrolidone-co-Methyl Methacrylate poly(MMA-co- t-BOC-NVP) and *tert*-butyl methacrylate-co- N-vinyl-2-pyrrolidone poly(*t*-BMA-co-NVP) in different compositions using free radical polymerization. Due to its hydrophilic and good biocompatibility character, N-Vinyl-2-pyrrolidone was used in the above polymer systems.

EXPERIMENTAL

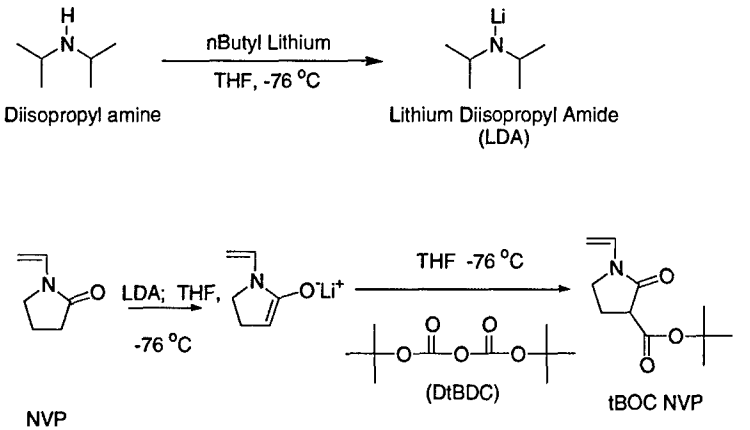
Materials. Reagents used for monomer and polymer synthesis were obtained from Acros and/or Aldrich. Dissopropylamine and tetrahydrofuran (THF) were dried over sodium metal overnight using benzophenone as an indicator and distilled prior to use. *N*-Vinyl-2-Pyrrolidone (NVP) was dried over molecular sieves overnight and vacuum distilled prior to use.N, N'-Azobisisobutyronitrile (AIBN) was purified by recrystallization from methanol.

Triphenyl sulfonium hexafluoroantimonate (TPSHA), was used as a photo acid generators (PAG) for resist formulation. A 50 wt% TPSHA in PGME solution was purchased from Polysciences. Other reagents were of pure grade and used as received.

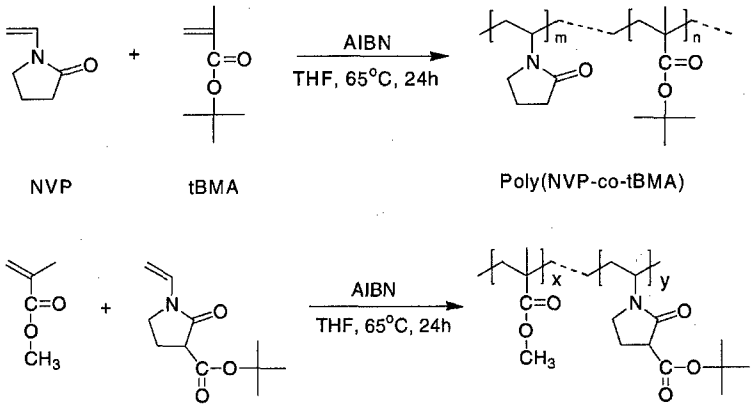
Synthesis of *t*-BOC-NVP. 3-(*tert*-butoxycarbonyl)-*N*-vinyl-2-pyrrolidone (*t*-BOC-NVP) was synthesized according to the literature [6]. N-butyl lithium (40 mL, 0.100 mol, 2.5 M solution in hexane) was added dropwise to diisopropylamine (14 mL, 0.100 mol) in anhydrous THF (40 mL) at –76 °C. After the addition was complete, the resulting solution (lithium diisopropylamide) was stirred at this temperature for 30 min and allowed to warm to room temperature. Thereafter, *N*-Vinyl-2-pyrrolidone (10.70 mL, 0.100 mol) was added dropwise to the suspension of lithium diisopropylamide at –76 °C and stirred at this temperature for 2 hrs. Di-*tert*-butyl dicarbonate (24 g, 0.100 mol) in 20 mL THF was added dropwise, to the reaction mixture and stirred at –76 °C for 2 hr. A saturated solution of NH_4Cl was added to the resulting solution after it had warmed to room temperature. This was done to quench the reaction. The organic layer was separated and the aqueous layer was extracted with diethyl ether. The combined organic solution was washed several times with deionized water and dried over anhydrous $MgSO_4$. It was then concentrated using a Büchi rotary evaporator and the product purified by column chromatography on silica gel with hexanes/ethyl acetate (4/1) as eluent. A 9.6 g of 3-(*tert*-butoxycarbonyl)-*N*-vinyl-2-pyrrolidone (*t*-BOC-NVP) was obtained as a colorless liquid (76% yield).

^{1}H NMR ($CDCl_3$: δ 7.0 (1H, q), δ 4.5 (2H, m), δ 3.4-3.7 (1H at carbon number three (C3) and carbon number five (C5) of the lactam ring), δ 2.2-2.5 (2H on carbon number four (C4) of the lactam ring), δ 1.4 (9H, s, tert-butyl); FTIR (KBr, cm $^{-1}$): 2980 (cyclic CH), 1733 (ester carbonyl), 1704 (amide carbonyl), 1634 (vinyl), 1153 (tert-butyl)

Synthesis of *t*-BOC-NVP-co-MMA and *t*-BMA-co-NVP. A series of copolymers of *t*-BOC-NVP with NVP and t-BMA with NVP were prepared in THF solutions using AIBN as free radical initiator at 65 °C, in sealed glass ampoules under vacuum. The polymers were isolated by precipitating in Hexane solution. The precipitates were purified by washing them several times with Hexane solution and dried under vacuum for 24hr **(Scheme 1)**



Synthetic Scheme 1



Synthetic Scheme 2

Polymer Surface Microfabrication. Photoresist solutions were prepared by dissolving a polymer and triarylsulfonium hexafluoroantimonate used as a photoacid generator in cyclohexanone or propylene glycol methyl ether. It was then spin-coated at 2000 rpm on a microscope slide glass and baked at 90°C to remove the solvent. Exposures were done through two types of masks (25 μm line by 25 μm space and 25 μm line by 50 μm space) on a Contact Mask Printer. The exposed samples were immediately baked at 115°C or a temperature below the glass transition temperature (Tg) of the polymer on a hot plate and washed in deionized water for 60 s to reveal the patterns as shown in **Figure 1a** & **1b**.

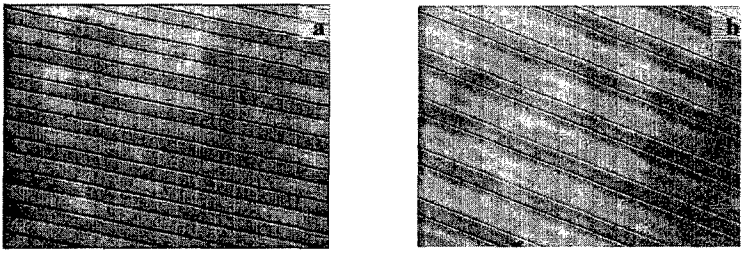


Figure 1: (a) 25 μm line by 50 μm space pattern ;b) 25 μm line by 25 μm space pattern

Cell Assay and Culture. Fibroblast cells were cultured on patterned surfaces. Glass slides with patterns were sterilized by rinsing with sterile Phosphate Buffered Saline (PBS) several times and placed in 1-well Lab-Tek chambered coverglass tissue culture plates, which were precoated with poly (2- hydroxyehtyl methacrylate). Cells were washed with serum-free medium before seeding onto the samples at a density of 1.0×10^5 cells/well. Non-patterned scaffold is used as a control. Cell cultures were carried out in a serum media. After seeding, fibroblast cells are allowed to stay in contact with the

surfaces in an incubator at 37 °C with 8% CO_2 for various periods of time. At the end of each incubation period, the samples are rinsed with PBS to remove nonattached cells.

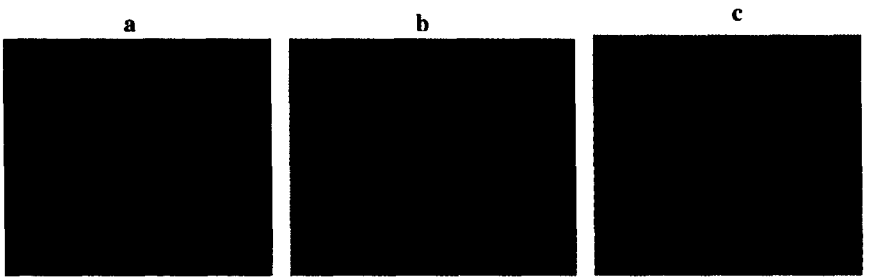


Figure 2: Confocal Fluorescence Microscope Images of, (a) Fibroblast cell seeded on 25 μm/25 μm L/S pattern; (b) & (c) Fibroblast cell seeded on 25 μm/50 μm L/S pattern.

RESULTS AND DISCUSSION

Polymer	Feed Ratio (mole ratio)		Composition (by ^{1}H NMR)		M.wt (Daltons)	Tg/°C
	TBNVP	MMA	TBNVP	MMA		
F1	0.65	0.35	0.31	0.69	16,085	100.12
F2	0.59	0.41	0.44	0.56	21,682	122.71
F3	0.42	0.58	0.14	0.86	18,038	103.10
F4	0.32	0.68	0.11	0.89	23,131	105.86
F5	0.24	0.76	0.21	0.80	19,435	101.73
F6	0.14	0.86	0.10	0.90	21,895	
F7	0.10	0.90	0.09	0.91	24,035	96.79

Table1. Combinatorial Matrix for *t*-BOC-NVP-co-MMA synthesis

Polymer	Feed Ratio (mole ratio)		Composition (by ^{1}H NMR)		M.wt (Daltons)	Tg/°C
	NVP	TBMA	NVP	TBMA		
Y1	0.80	0.20	0.83	0.17	9,021	111.00
Y2	0.60	0.40	0.69	0.31	13,260	123.55
Y3	0.40	0.60	0.59	0.41	10,939	93.66
Y4	0.20	0.80	0.15	0.85	10,639	85.51
Y5	1.00	0.00	1.00	0.00	NA	67.75
Y6	0.00	1.00	0.00	1.00	3,294	NA
Y7	0.70	0.30	0.89	0.11	15,537	88.65
Y8	0.50	0.50	0.71	0.29	15,492	103.50
Y9	0.30	0.70	0.51	0.49	17,362	104.48
Y10	0.10	0.90	0.55	0.45	19,055	73.06
Y11	0.90	0.10	0.86	0.14	10,091	129.31

Table 2 . Combinatorial Matrix for *t*-BMA-co-NVP synthesis

From **Table 1,** we observed that all poly(t-BOC-NVP-co-MMA) polymer systems supported cellular growth and activities. This we attributed to the protecting t-BOC group attached to the NVP. Once the t-BOC group is exposed to UV light, it becomes very hydrophilic and hence supports cell growth and proliferation. The higher the percentage composition of t-BOC-NVP groups in the polymer, the higher the number of cellular activities and proliferation. For the poly (t-BMA-co-NVP) polymer system, **Table 2,** polymers with high percentage composition of NVP were found to be water soluble. NVP is very hydrophilic and hence absorbs water making the polymer soluble in aqueous environment.

The solubility of the polymers in aqueous environment was used as a criterion for the rapid screening of the polymers for scaffold fabrication. Water soluble polymers will dissolve once seeded with cells in an aqueous media. These types of polymers were therefore not used for scaffold fabrication.

On the patterned polymer surface, one region has hydrophilic (adhesion) patterns whereas another had hydrophobic (non-adhesion or inhibited) patterns. When cells were cultured on the patterned surfaces, preferential cell adhesion to the hydrophilic area was obtained. Cells were also found to elongate and align along the hydrophilic grooves. This region is rich in carboxylic acid groups and the hydrophilic proteins or integrins in the serum are preferentially adsorbed on to it. The cells then adhere to this surface through an integrin/ligand interaction. The elongation and alignment of the cells along the hydrophilic grooves can be explained by a phenomenon known as "contact guidance". Contact guidance occurs when a cell on a given substratum assumes a corresponding orientation and moves along that line.

Topographical features on substratum surfaces that affect cell orientation and alignment includes groove depth, width and ridge (space between two grooves). Depth is the most important effect in determining cell orientation. Orientation increases with increase depth, but decreases with increasing width. From **Figure 3,** the groove depth in this study was found to be between 200 and 250 nm. It has been shown that cells such as fibroblasts react to depth as shallow as 70nm [7]. Ridge distance was also found to be an important factor for cell orientation and alignment. From **Figure 2a**, cells seeded on the patterned polymer surfaces were occasionally observed to reach out to their adjacent neighbors. This was due to the short distance, 25μm, between the patterned lines or grooves where cells bridge across the patterns and form a confluent layer on top of the pattern. Such contact is avoided by increasing the distance between the patterns 50μm, **Figure c & d.**

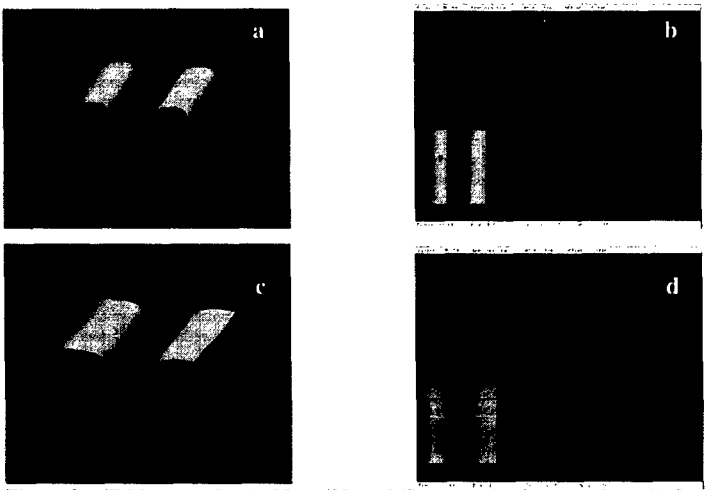


Figure 3: AFM Images of, (a) 25 μm/25 μm L/S patterned polymer surface; (b) Section Analysis with a step height or groove of 207 nm: (c) 25 μm/50 μm L/S patterned polymer surface; (d) Section Analysis with a step height or groove of 244 nm

CONCLUSION

Combinatorial approach for the design of two sets of polymer having small but systematic structural variations can lead to the rapid preparation of extensive polymer systems. It also has the potential of identifying correlation between chemical structure of polymeric scaffolds and cellular response.

These results also imply one potential application of using this technique in combination with 3-D bioresorbable constructs to produce an oriented tissue-like structure from fibroblasts, which will have desirable mechanical strength and flexibility similar to that of normal tissue.

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